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The critical DNA flanking sequences of a CpG oligodeoxynucleotide, but not the 6 base CpG motif, can be replaced with RNA without quantitative or qualitative changes in Toll-like receptor 9-mediated activity

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Abstract

Double- and single-stranded oligodeoxynucleotides containing unmethylated cytosine–guanosine (CpG) dinucleotides (CpG-ODN) activate immune cells via TLR9. In this report we synthesized hybrid DNA–RNA molecules (HDR) in order to further explore the structure–immune function relationship of CpG-ODN in TLR9 signaling and the potential immunomodulatory properties of RNA. We demonstrate that replacement of the deoxyadenosine flanking sequences, critical for the immune activating properties of CpG-ODN, with a similar number of adenosines, although not guanosines, cytosines, or uracils, maintains complete immunostimulatory activity of the hybrid oligonucleotide in vitro, whereas a similar RNA replacement of even 1 base of the required unmethylated 6 base DNA motif (purine–purine–CpG–pyrimidine–pyrimidine) results in a complete loss of activity. Regardless of whether the critical flanking sequence was RNA or DNA there was no significant change in the quantitative or qualitative immune-stimulating activity, or TLR-specificity of the resulting sequences, thus underscoring the relatively permissive functional role of the flanking sequence, and the more specific role of the motif in mediating TLR9 signaling. These data further support a potential role for RNA in immunomodulation.
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Keywords: CpG; Oligonucleotides; RNA; DNA; Adjuvant; Humoral immunity

1. Introduction

Both double- and single-stranded bacterial or synthetic DNA are capable of inducing immune activation via TLR9 [1]. The immunostimulatory properties of DNA depend on the presence of unmethylated cytosine–guanosine dinucleotides and appropriate flanking sequences (CpG-oligodeoxynucleotide [ODN]) present naturally at higher frequencies within microbial, as opposed to mammalian, DNA [2,3]. Although unmethylated CpGs are critical for both human and mouse TLR9-mediated signaling, distinct flanking sequences are required for CpG-ODN[1] activity on mouse versus

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[1] *Abbreviations used:* CpG-ODN, cytosine–guanosine dinucleotide containing oligonucleotide; HDR, hybrid DNA–RNA; ssRNA, single-stranded RNA; PspA, pneumococcal surface protein antigen; anti-IgD-Dex, dextran conjugated anti-mouse-IgD antibody; TLR, Toll-like receptor.

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14. ABSTRACT

Double- and single-stranded oligodeoxynucleotides containing unmethylated cytosine?guanosine (CpG) dinucleotides (CpGODN) activate immune cells via TLR9. In this report we synthesized hybrid DNA?RNA molecules (HDR) in order to further explore the structure?immune function relationship of CpG-ODN in TLR9 signaling and the potential immunomodulatory properties of RNA. We demonstrate that replacement of the deoxyadenosine Xanking sequences, critical for the immune activating properties of CpG-ODN, with a similar number of adenosines, although not guanosines, cytosines, or uracils, maintains complete immunostimulatory activity of the hybrid oligonucleotide in vitro, whereas a similar RNA replacement of even 1 base of the required unmethylated 6 base DNA motif (purine?purine?CpG?pyrimidine?pyrimidine) results in a complete loss of activity. Regardless of whether the critical Xanking sequence was RNA or DNA there was no signiWcant change in the quantitative or qualitative immunestimulating activity, or TLR-speciWcity of the resulting sequences, thus underscoring the relatively permissive functional role of the Xanking sequence, and the more speciWc role of the motif in mediating TLR9 signaling. These data further support a potential role for RNA in immunomodulation.

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human cells, based on the divergent structure of the TLR9s in the two species. In the mouse, a 6 bp "motif" (i.e., purine–purine–CpG–pyrimidine–pyrimidine) is required for immunostimulation, along with additional flanking deoxynucleotides [2,4].

Many viruses direct the synthesis of double-stranded (ds)RNA within infected host cells. dsRNA and its synthetic counterpart, polyinosinic–polycytidylic acid (poly[I:C]) can activate the innate immune system via Toll-like receptor (TLR)3 [5]. The ability of TLR3 to mediate induction of proinflammatory cytokines, including type I IFN suggests a role for this TLR in cell-mediated immunity to viruses and other intracellular pathogens [5]. During an immune response to a microbial pathogen it is also likely that single-stranded (ss)RNA, released via pathogen lysis and by necrotic host cells, also comes into contact with cells of the innate immune system. However, in contrast to dsRNA, single-stranded or denatured RNA fails to activate immune cells via TLR3 [5]. Recently two papers appeared which implicated TLR7 (mouse) and/or TLR8 (human) in cell signaling in response to single-stranded (ss)RNA. Diebold et al. [6] reported that ssRNA from influenza virus or synthetic poly U induced cytokines in both plasmacytoid and $CD11b^{+}B220^{-}$ murine DC via TLR7 in a MyD88-dependent fashion. Heil et al. [7] demonstrated that guanosine and uridine rich ssRNA from HIV-1 stimulated cytokine production from both murine plasmacytoid DC and macrophages.

In light of the above findings, we synthesized hybrid DNA–RNA molecules (HDR) in order to further explore the structure–immune function relationship of CpG-ODN in TLR9 signaling and the potential immunomodulatory properties of RNA. We show, for the first time, that RNA can replace the critical DNA flanking sequences in the single-stranded CpG-ODN, but not the 6 base CpG motif, without quantitative or qualitative changes or TLR9-specificity, for induction of murine immune cell activation in vitro and adjuvanticity in vivo. These data thus underscore the relatively permissive functional role of the flanking sequence, and the more specific role of the motif in mediating TLR9 signaling by CpG-ODN, and further support a potential role for RNA in immunomodulation.

2. Materials and methods

2.1. Mice

Specific pathogen-free (SPF) female BALB/c and C57BL/6 mice were obtained from the National Cancer Institute (Frederick, MD, USA). $TLR9^{-/-}$ [1] and $MyD88^{-/-}$ [8] mice were obtained from Dr. Shizuo Akira (Osaka University, Osaka, Japan). Mice were maintained under SPF conditions and were used at 8–10 weeks of age.

2.2. Synthesis of HDR

Syntheses of phosphorothioated hybrid DNA–RNA (HDR) molecules were performed on an Applied Biosystems 394 DNA/RNA Synthesizer (ABI, Foster City, CA, USA). The DNA Phosphoramidites were purchased from Applied Biosystems, and the RNA Phosphoramidites and RNA columns were purchased from Glen Research (Sterling, VA, USA). Our 394-phosphoramidite arrangements had the DNA precursors placed in positions 1–4, while the RNA precursors were at positions 5–8. Thiolation was accomplished using Beaucage Reagent also purchased from Glen Research. The general matrix of the synthesis program used was the 1.0 μM Sulfur program that is resident to the ABI 394, however numerous step additions and time changes were made to increase the synthesis efficiency of the chimeric HDRs. Several examples of these are: (1) In their resident 394 program at the thiolation step, the step time is 900 s (the program is assuming you are using TETD for sulfurization); this was changed to 90 s in deference to the much faster addition of the sulfur by the Beaucage reagent. (2) The step time of the nucleotide addition was increased from 25 to 750 s to compensate for the slower coupling rate of the sterically hindered RNA bases. (3) The capping time was doubled from 5 to 10 s. (4) Several extra "wash" steps with acetonitrile were added after sulfurization, capping, and detritylation to ensure all reagents had been removed completely. All wash steps were increased in time by an additional 5–10 s for complete chemical removal.

Following synthesis completion, the HDRs were cleaved from the CpG using a 3:1 solution of ammonium hydroxide:ethanol and the 394's resident "End RNA" program. The HDRs were further deprotected for 12 h at 55 °C and then dried completely. To remove the silyl group from the 2′ hydroxyl of the RNA bases 500 μl of tetrabutylammonium fluoride (TBAF) in tetrahydrofuran (THF) was added to the dried HDR and agitated gently for 20 h at room temperature. The TBAF/THF was then diluted 1:6 using sterile, RNAse-free water and desalted using the Poly-Pak II Barrel from Glen Research and a modified desalting procedure to ensure complete removal of the TBAF/THF. After desalting an aliquot was taken from each HDR for optical density measurement and further characterized by HPLC and PAGE analyses. The remainder was frozen and lyophilized.

2.3. Synthesis of CpG-ODN

Synthesis of standard phosphorothioated DNA was accomplished using a copy of the resident 1.0 μM sulfur synthesis program with the only change in the oxidation step. Beaucage reagent was used for thiolation instead of TETD, therefore the thiolation step time was changed from 900 to 90 s. Following synthesis, the oligo was cleaved using the standard CE cleavage program in

concentrated ammonium hydroxide. The oligo was deprotected for 12 h at 55 °C and then lyophilized.

2.4. Proliferation assay

Spleen cell suspensions were depleted of RBCs using ACK lysing buffer (Quality Biological, Gaithersburg, MD, USA), and resuspended in culture medium (RPMI-1640 supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 0.05 mM 2-ME, 50 μg/ml penicillin, and 50 μg/ml streptomycin). Spleen cells (2×10^5 cells per well, in triplicate) were cultured in 96-well plate in the presence of different concentrations of HDR, CpG-ODN, or control oligonucleotide, at 37 °C in a 5% CO_2 incubator for 48 h. Thirty-seven kilobecquerel of [^{3}H]thymidine was added to each well and incubated for another 16 h followed by harvesting of the cells onto glass fiber filter membranes. [^{3}H]Thymidine incorporation was measured using a micro beta scintillation counter (Richard Allen Scientific, Kalamazoo, MI, USA). Data are presented as means cpm ± SEM.

2.5. Cytokine-specific ELISA

The concentrations of specific cytokines released into the media of spleen cell cultures were measured by optimized standard sandwich ELISA. Recombinant cytokines used as standards, as well as the capture mAbs, biotinylated mAbs used for detection, and streptavidin-alkaline phosphatases were purchased from BD Pharmingen (San Jose, CA, USA). Streptavidin-alkaline phosphatase was used in combination with trinitrophenyl phosphate (TNPP, Sigma, St. Louis, MO, USA) as a substrate to detect specific antibody binding. Standards were included in every plate and the samples were tested in duplicate. The limits of detection of the respective ELISAs were as follows: IL-6, 4 pg/ml; IL-10, 150 pg/ml; IL-12 (p40/70), 6 pg/ml. In brief, spleen cells (5×10^6 cells per well) from above preparation were cultured in 24-well plate in the presence of different concentration of HDR, CpG-ODN or control oligonucleotide at 37 °C in 5% CO_2 incubator for 48 h. Supernatants from these wells were then collected for measurement of cytokine concentrations. Ninety-six-well plate was coated with anti-cytokine antibody overnight at 4 °C, followed by non-specific blocking of the plate with 2% BSA in PBS for 2 h at room temperature. Culture supernatants containing the cytokines were added, incubated for 2 h at 37 °C, washed with PBS–Tween 20, incubated with biotin-labeled anti-cytokine antibody for 1 h, and washed followed by another incubation with streptavidin-alkaline phosphatase. Specific cytokine was detected using TNPP substrate solution. The plate was read at 405 nm on a multiskan ascent plate reader (ThermoLab systems, Finland) and cytokine concentrations were calculated from standard curves using purified cytokine.

2.6. Analysis of cell surface binding and internalization of HDR, CpG-ODN or control oligonucleotide

Spleen cell (1×10^6/ml) suspensions were prepared as stated earlier and cultured in medium at 37 °C for 30 min. FITC-labeled HDR, CpG-ODN, or control oligonucleotide (1 μM) was added to the cell suspension and incubated at 37 °C for various periods of time followed by addition of ice-cold PBS to stop the binding and internalization. Washed cells were analyzed by FACS for total binding. Ice cold 2% trypan blue was then added, and cells were re-analyzed to detect internalized ODN [9]. Cells incubated on ice for 125 min with labeled ODN and cells cultured on ice for 30 min with labeled ODN, followed by washing with cold PBS, served as negative controls.

2.7. Immunization of mice with antigen and HDR

BALB/c mice (female, 8- to 10-week old, 7 per group) were immunized intraperitoneally with 0.5 μg PspA (pneumococcal surface protein A) and 25 μg HDR, CpG-ODN, or control oligonucleotide adsorbed on 13 μg alum, followed by boosting on day 28 with the same preparations. Serum samples for measurement of antigen-specific Ig isotype titers were prepared from blood obtained through tail vein on day 35. Recombinant PspA was expressed in *Saccharomyces cerevisiae* BJ3505 as a His_6-tagged fusion protein, and purified by Ni–NTA affinity chromatography as previously described [10].

2.8. Measurement of serum antigen-specific Ig isotype titers

Immulon 4 plates were coated with PspA (5 μg/ml) in PBS overnight at 4 °C. Plates were then blocked with blocking buffer (2% BSA in PBS) for 1 h at room temperature (RT). Threefold dilution of serum samples in blocking buffer was then added starting at a 1:50 serum dilution. After 2 h incubation at RT, plates were washed three times with PBST (PBS with 0.05% Tween 20). Alkaline phosphatase conjugated polyclonal goat anti-mouse IgG, IgG1, IgG2a, IgG2b, and IgG3 (Southern Biotec, AL; final concentration in blocking buffer, 200 ng/ml) were added and incubated for another 1 h at RT. Plates were washed five times with PBST and bound antibodies were developed using TNPP substrate solution. The plate was read at 405 nm and antibody titers were calculated from a standard curve using any single serum dilutions and corresponding OD_{405nm}. Data are presented as geometric mean of seven mice serum.

2.9. Statistics

Data are presented as the arithmetic means (triplicate) of cpm of individual cultures plus or minus the

standard errors of the means (SEM). For antibody titers, data are presented as the geometric means of Ig titers of seven serum samples plus or minus SEM. Differences between treatment groups were considered significant at P values of <0.05 using the Student's t test.

3. Results and discussion

3.1. Selective substitution of the CpG-ODN flanking sequences with an RNA polyadenosine maintains induction of proliferation and cytokine release by spleen cells

The ability of CpG-ODN to activate mouse cells has been shown to depend on a particular 6 base DNA motif (AACGTT) and a critical number of additional flanking nucleotides, whose particular sequences are less conserved [11]. We initially asked whether a change, from DNA to RNA, in the nucleotide sequences flanking the 6 base CpG motif would maintain or change the nature of the stimulatory activity of the CpG-ODN on mouse cells. We thus synthesized a series of oligonucleotides with a phosphorothioate-modified backbone in which the 6 base CpG DNA motif was flanked on each side with six ribonucleotide adenines, guanines, cytosines or uracils (see Table 1). These hybrid DNA–RNA (HDR) molecules were added to spleen cell cultures at various concentrations and cellular proliferation, as reflected by [^{3}H]thymidine incorporation, was measured during the interval between 48 and 64 h following initiation of culture. As demonstrated in Fig. 1A, the 18-mer HDR with polyadenosine flanking the core CpG motif ("18a"), but not those with poly "g", "c" or "u" comprising their flanking sequences, stimulated spleen cell proliferation in a dose-dependent manner, quantitatively similar to the 18-mer CpG-ODN possessing a polydeoxyadenosine flanking sequence ("18 A"). We further assessed the ability of these HDR to induce cytokine synthesis. As illustrated in Fig. 1B, HDR 18a, but not 18 g, 18c or 18 u induced in spleen cell cultures, the proinflammatory cytokines IL-6 and IL-12, and the anti-inflammatory cytokine IL-10 in a dose-dependent manner. The effect of the 18a HDR was quantitatively and qualitatively similar to that of the 18A CpG-ODN. We also observed that the 18a HDR selectively upregulated class II-MHC and CD86 expression on splenic B cells comparable to that observed with CpG-DNA (data not shown).

Table 1
Sequences of RDR used in our study

Name	Sequences
8a	a AACGTT a
12a	aaa AACGTT aaa
18a	aaa aaa AACGTT aaa aaa
24a	aaa aaa aaa AACGTT aaa aaa aaa
30a	aaa aaa aaa aaa AACGTT aaa aaa aaa aaa
8A	A AACGTT A
12A	AAA AACGTT AAA
18A	AAA AAA AACGTT AAA AAA
24A	AAA AAA AAA AACGTT AAA AAA AAA
30A	AAA AAA AAA AAA AACGTT AAA AAA AAA AAA
18g	ggg ggg AACGTT ggg ggg
18c	ccc ccc AACGTT ccc ccc
18u	uuu uuu AACGTT uuu uuu
1502 (suppressive)	GAG CAA GCT GGA CCT TCC AT
30a-CG	aaa aaa aaa aaa CGCGCG aaa aaa aaa aaa
30a-GC	aaa aaa aaa aaa AAGCTT aaa aaa aaa aaa
30a-CG only	aaa aaa aaa aaa aaCGaa aaa aaa aaa aaa
30a-4B	aaa aaa aaa aaa aACGTt aaa aaa aaa aaa
30a-RRR	aaa aaa aaa aaa aacgtt aaa aaa aaa aaa

Capital letters denote "DNA" and small letters denote "RNA."

3.2. The length of the flanking polyadenosine is a critical determinant in HDR-mediated stimulatory activity

In the next experiment, we wished to determine the optimal length of the polyadenosine flanking sequence required for full HDR activity. We synthesized a series of HDR and CpG-ODN each containing the 6 base DNA motif, but differing in the number of flanking adenosines or deoxyadenosines. As illustrated in Fig. 2, 18a, 24a, and 30a HDR were each optimally potent mitogens for spleen cell cultures, with progressively lower but still statistically significant mitogenic activity observed for 12a and 8a. The mitogenic responses to 18a, 24a, and 30a were not statistically different. In contrast, 24A and 30A CpG-ODN each showed optimal mitogenic activity, with progressively and significantly lower activity observed for 18A and 12A, and no significant effect was observed for 8A.

3.3. HDR synergizes with multivalent membrane Ig crosslinking signals for B cell proliferation and cytokine secretion

It was previously demonstrated that CpG-ODN synergizes with BCR crosslinking signals for induction of proliferation and IL-6 secretion [2,12]. In this regard, we utilized dextran-conjugated anti-IgD antibodies (anti-IgD-dex) to compare the ability of HDR and CpG-ODN to synergize with signals mediated through multivalent BCR crosslinking. Several suboptimal concentrations of anti-IgD-dex were used in combination with a suboptimal amount of 30a HDR. 30a HDR demonstrated its ability to synergize for B cell proliferation (Fig. 3A). Anti-IgD-dex alone failed to induce either detectable IL-6 or IL-12 secretion from spleen cell cultures, but synergistically enhanced the IL-6-inducing ability of 30a HDR (Fig. 3B), which is statistically significant compared to anti-IgD-dextran alone. Anti-IgD-dex alone also failed to induce IL-12, but in

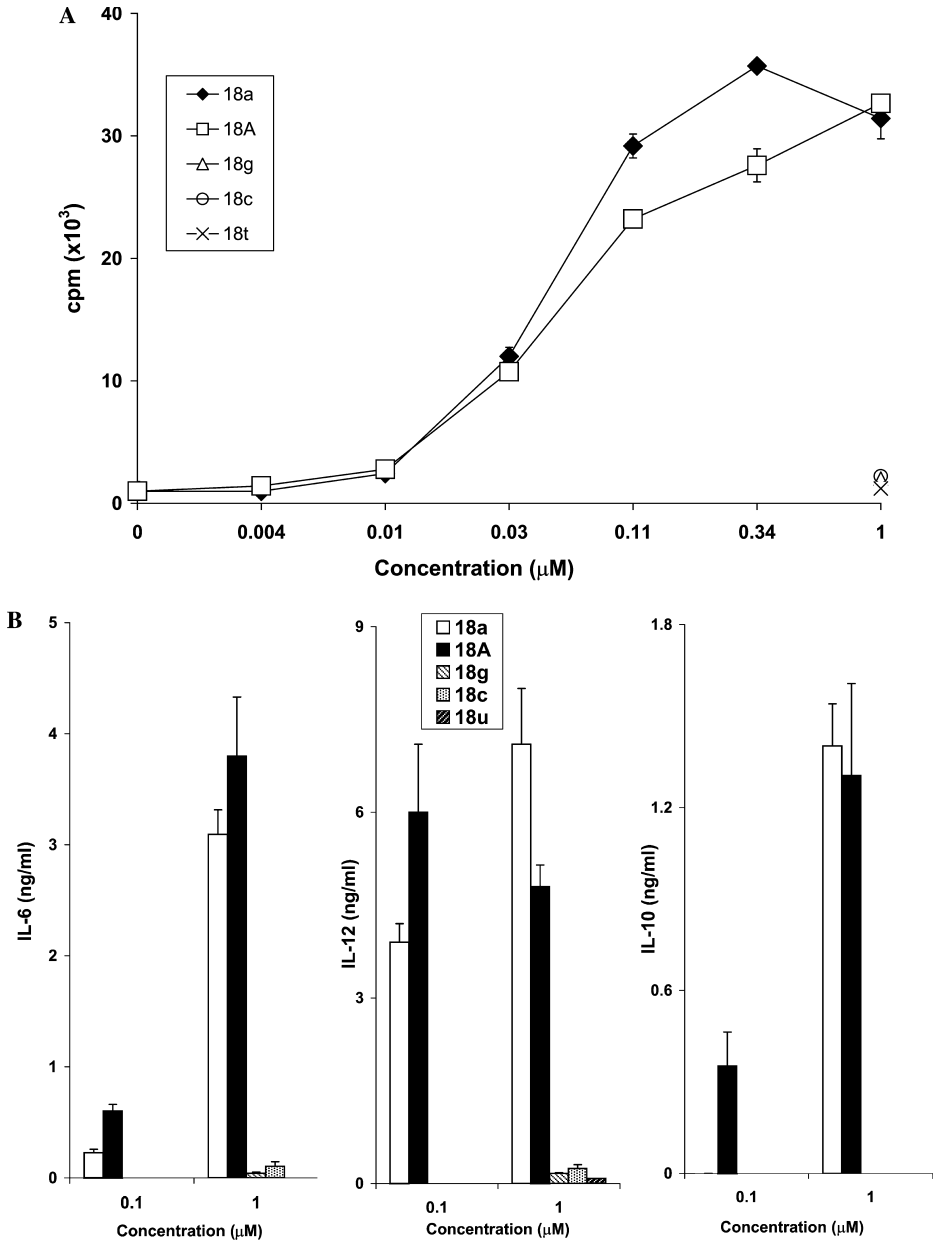

Fig. 1. Effects of HDR on in vitro murine splenocyte proliferation and cytokine release. BALB/c spleen cells were incubated for 48 h with different concentrations of HDRs and CpG-ODN and then pulse-labeled with [^{3}H]thymidine (A). For cytokine release assay, after 48 h culture, supernatants were assayed for the presence of IL-6, IL-10, and IL-12 (B). Results are means ± SEM of triplicate cultures and are representative of three experiments.

contrast to IL-6, did not synergize with 30a for IL-12 induction. 30A CpG-DNA also synergize B-cell proliferation and IL-6 production comparably to 30a HDR whereas, control 30-mer HDR does not (data not shown).

3.4. The 6 base CpG-ODN motif is necessary for HDR stimulatory activity

The hexameric CpG "motif" is critical for the stimulatory activity of CpG-ODN that is active on murine immune cells [11,13]. So far, we demonstrated that the 6-mer CpG DNA motif when flanked by a sufficient number of RNA polyadenosines can activate mouse spleen cells in a manner quantitatively and qualitatively similar to the full-length CpG-ODN. We next wished to determine whether the 6 base motif would continue to mediate activity when one or more of its DNA bases were substituted with an analogous RNA base. We thus synthesized a series of 30-mer HDR, containing polyadenosine flanking sequences, with modifications in this 6 base DNA core sequence, and determined their ability to induce spleen cell proliferation. Substitution of all six deoxynucleotide bases of

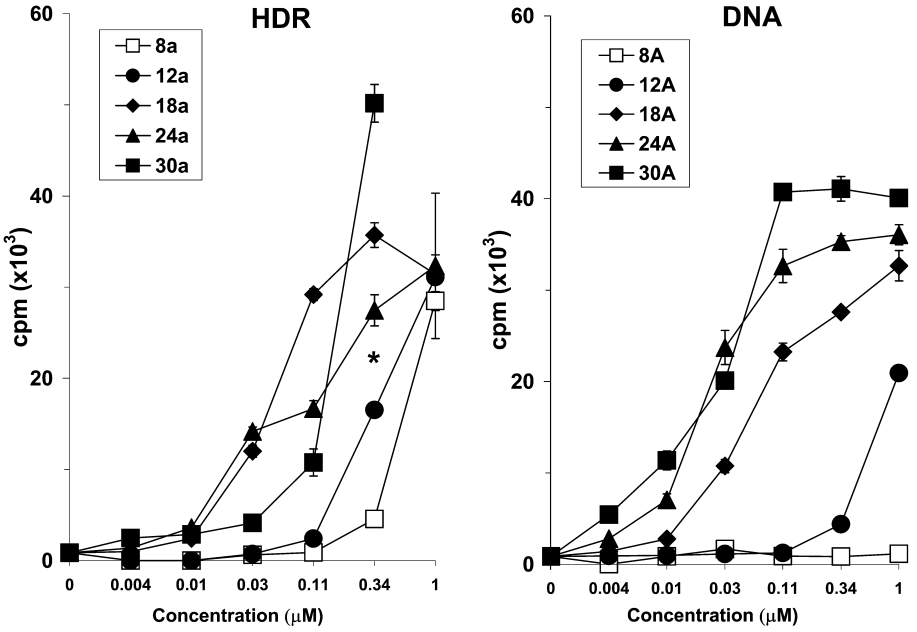

Fig. 2. Flanking length of HDRs to the CpG-motif is important for their stimulatory activity. HDRs and CpG-ODN of varying length were synthesized and BALB/c spleen cells were incubated for 48 h with varying concentrations of these synthetic molecules, followed by pulse-labeled with [^{3}H]thymidine. Results are means ± SEM of triplicate cultures and are representative of three experiments. *$P < 0.003$, between 12a and 18a.

the motif with corresponding RNA bases resulted in a complete loss of mitogenic activity (Fig. 4). Likewise, a similar substitution in the single most 5′ and 3′ bases of the motif also resulted in a non-stimulatory HDR. Additional changes in the motif, such as a change of the central CG to GC, or in the bases flanking the unmethylated CG similarly led to loss of activity. Moreover, these modified HDRs also failed to stimulate cytokine secretion or upregulation of MHC class II or CD86 expression on spleen cells (data not shown). Collectively these data illustrate the ability to maintain immunostimulation of CpG-ODN through substitution of the flanking sequences, but not the 6 base DNA core motif, with corresponding RNA bases.

3.5. Suppressive DNA inhibits both stimulatory CpG-ODN and HDR action

In the next series of experiments we wished to determine whether the mechanism of HDR action was similar to that of CpG-ODN. To begin we tested a synthetic DNA, having particular base sequences, previously shown to be capable of suppressing the stimulatory action of CpG-ODN [14]. Suppressor DNA can inhibit both CpG-ODN-mediated cytokine production in vitro, and reduced the adjuvant effects of CpG-ODN in vivo. We synthesized one suppressive DNA (sequence 1502) as reported previously [15] and examined its ability to inhibit HDR activity. Fig. 5 demonstrates that suppressive DNA 1502 exhibited a similar degree of inhibition of both HDRs and CpG-ODN-mediated proliferation and cytokine release (IL-6 and IL-12) in spleen cell cultures.

3.6. HDR stimulates immune cells through TLR9 and MyD88

CpG-ODN stimulates immune cells through TLR9 and the TLR adapter protein, MyD88 [1,8]. Although the mechanism by which TLR9 recognizes CpG-ODN is currently unknown, we assumed that the 6 base DNA motif was critical for TLR9 recognition given the strict requirement for this particular sequence, in contrast to the variety of possible flanking sequences, which allow for activity. In this regard we tested whether HDR likewise requires TLR9 for signaling. We thus stimulated spleen cells from wild type versus TLR9$^{-/-}$ mice with a 30-mer HDR versus CpG-ODN. Fig. 6A shows that both 30-mer HDR and DNA failed to stimulate proliferation in spleen cells from TLR9$^{-/-}$ mice, whereas LPS, a TLR4 ligand, stimulates both wild type and TLR9$^{-/-}$ spleen cells to a comparable degree. Likewise, both HDR and CpG-ODN failed to stimulate proliferation in spleen cells from MyD88$^{-/-}$ mice (Fig. 6B). LPS also failed to stimulate MyD88$^{-/-}$ spleen cell proliferation consistent with the critical role of MyD88 in TLR4, as well as, TLR9 signaling.

3.7. HDR and CpG-ODN are internalized with similar kinetics

Cell surface DNA binding receptors have been described, but appear to lack any sequence specificity, as stimulatory CpG-ODN and non-stimulatory ODN bind equally well to cell membranes [9,15]. ODN are internalized by lymphocytes through an active,

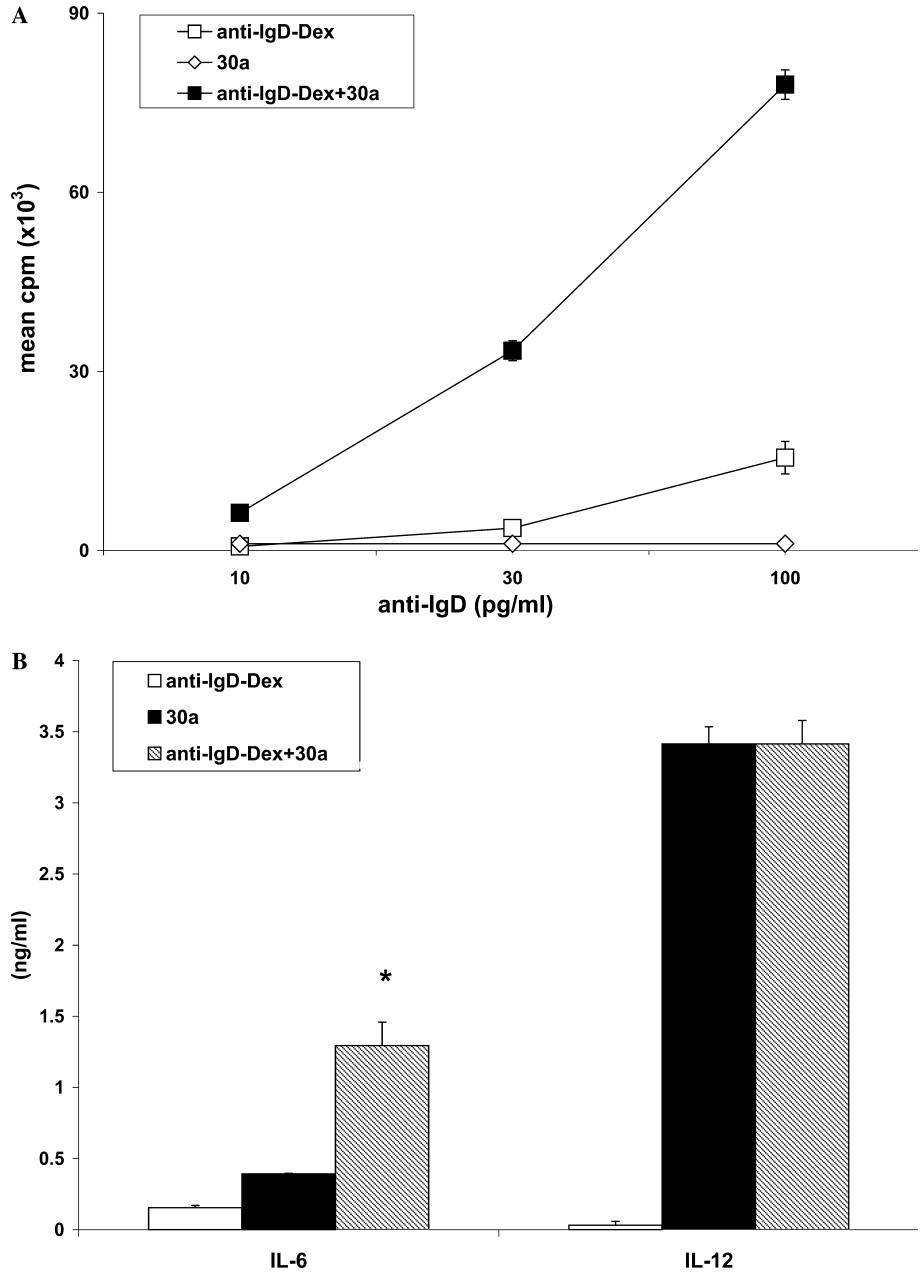

Fig. 3. Anti-IgD-dextran synergizes with HDR containing stimulatory CpG-motif for proliferation and cytokine release. BALB/c spleen cells were stimulated with suboptimal concentration (0.04 μM) of 30a HDR along with varying concentrations of anti-IgD-dextran (10, 30 or 100 pg/ml). [^{3}H]Thymidine was added 48 h after initiation of culture for a 16 h period and cultures were then harvested for [^{3}H]thymidine incorporation (A). For cytokine release assay, after 48 h culture, supernatants were assayed for the presence of IL-6 and IL-12 (B). Results are means ± SEM of triplicate cultures and are representative of three experiments. *$P < 0.006$, relative to anti-IgD-dextran alone.

temperature-dependent process, which is generally sequence-independent. We wished to determine whether the presence of RNA in the flanking sequences of HDR significantly alters its cellular binding and internalization, relative to CpG-ODN. We thus synthesized FITC-labeled HDR, CpG-ODN, and control HDR and determined their kinetics of internalization within spleen cells. Fig. 7 demonstrates that all the above three bind and internalize to spleen cells with similar kinetics.

3.8. HDR acts as a Th1-like adjuvant to antigen driven responses

Several groups reported that CpG DNA is potent Th1 type adjuvants for induction of Ig responses in vivo [16–19]. We thus wished to compare the ability of HDR versus CpG-ODN for their abilities to act as adjuvants when co-injected with a soluble protein antigen. BALB/c mice were injected i.p. with 0.5 μg of soluble, recombinant pneumococcal surface protein A (PspA) along with

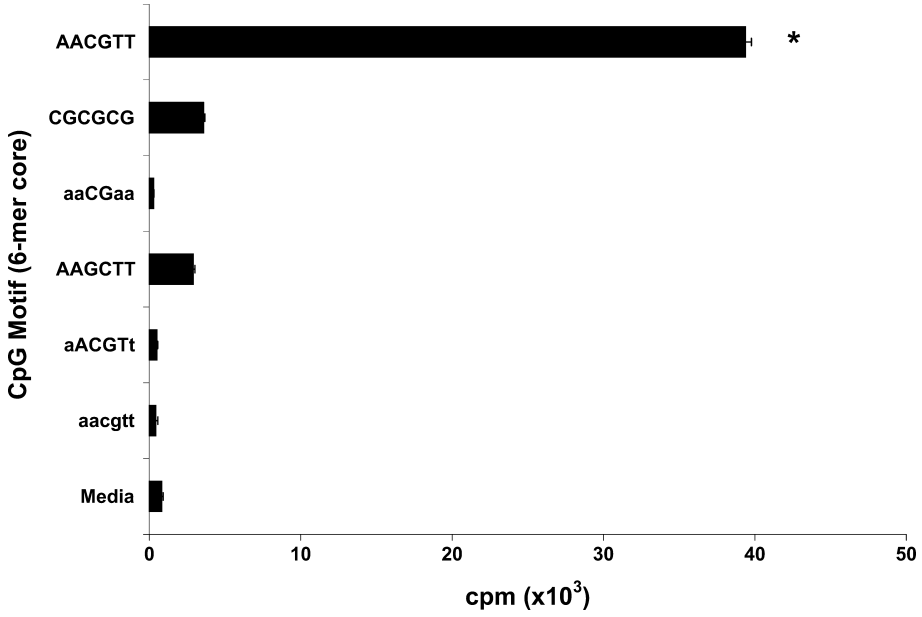

Fig. 4. The 6-mer CpG-motif is essential for these HDRs activity. BALB/c spleen cells were incubated for 48 h with a series of modified CpG-motif HDRs and then pulse-labeled with [^{3}H]thymidine for 16 h followed by harvesting for [^{3}H]thymidine incorporation. Results are means ± SEM of triplicate cultures and are representative of three experiments. *$P < 0.001$ relative to 6-mer motif with all RNA.

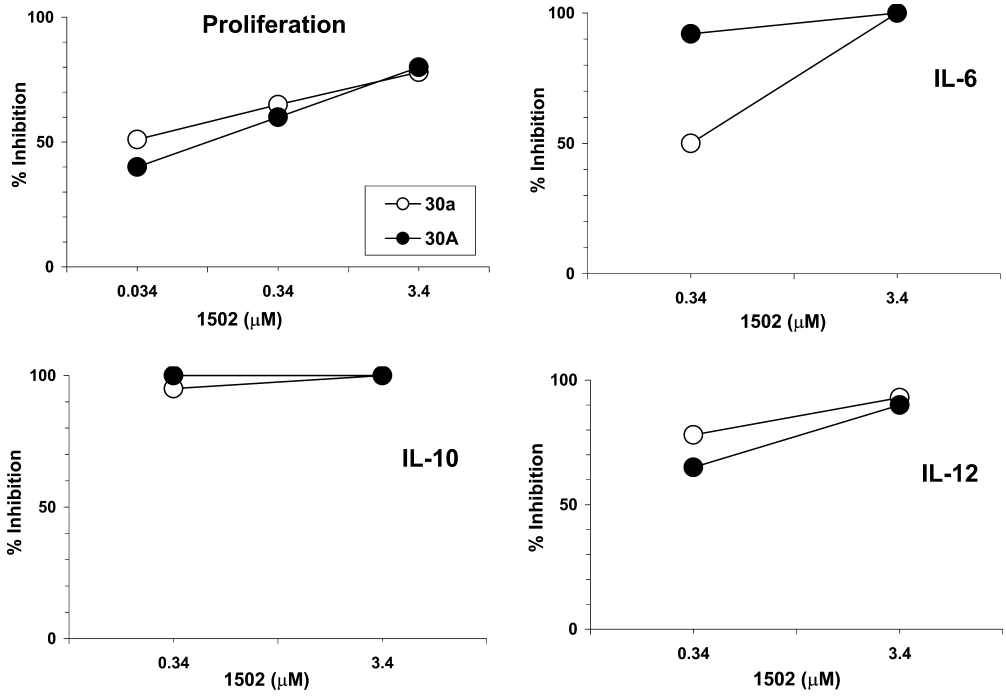

Fig. 5. Suppressor DNA can block HDR-mediated stimulation of immune cells. BALB/c spleen cells were stimulated with 0.34 μM concentration of 30-mer HDR or CpG-ODN in presence of increasing amount of suppressive DNA 1502 for 48 h followed by pulse with [^{3}H]thymidine for 16 h and [^{3}H]thymidine incorporation was measured and cytokine levels in culture supernatants were also measured.

25 μg of 24-mer HDR or CpG-ODN, and a relatively small amount of alum (13 μg) on days 0 and 28. Sera were collected 1 week after the second immunization and tested for the presence of PspA-specific Ig isotype antibodies by ELISA. Fig. 8 illustrates that the geometric mean titer of IgG anti-PspA antibody was significantly increased ($P < 0.05$) by 86-fold and 97-fold upon addition of 24-mer HDR or CpG-ODN, respectively, whereas a control 24-mer HDR, which fails to stimulate spleen cells in vitro, enhanced IgG anti-PspA titers only threefold. PspA-specific IgG3, IgG1, IgG2b, and IgG2a were all significantly enhanced ($P < 0.05$) by both HDR and CpG-ODN suggesting that they both function as a Th1 type adjuvant. Similar results were obtained when

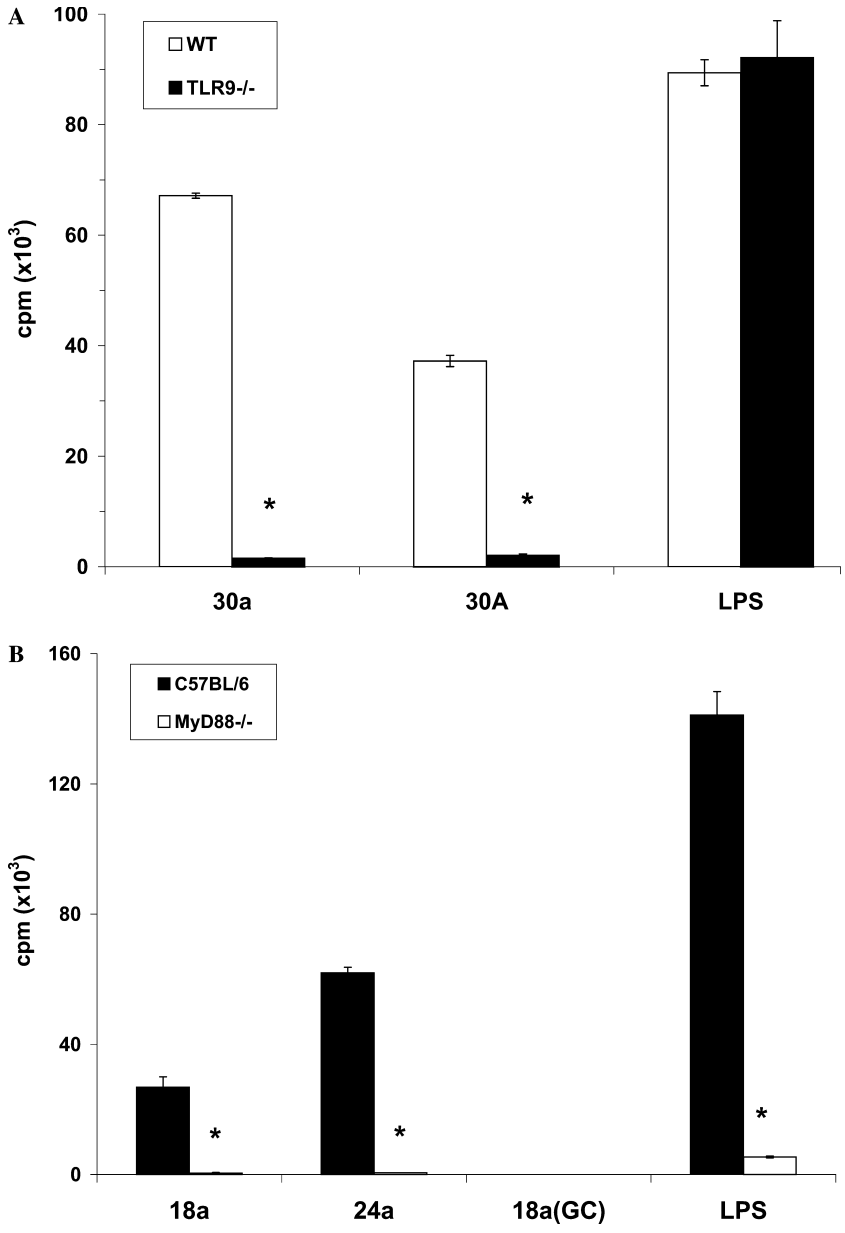

Fig. 6. HDR stimulates immune cells through Toll-like receptor 9 and adapter protein MyD88. Splenocytes from wild type or TLR9$^{-/-}$ and MyD88$^{-/-}$ mice were cultured with 1 μM concentration of different HDR, DNA and control HDR or LPS for 48 h, pulsed with [^{3}H]thymidine for 16 h and [^{3}H]thymidine incorporation was measured by beta scintillation counter. Data indicate means ± SEM of triplicate samples of one representative experiment (A and B). *$P < 0.01$, compared to proliferation of wild type spleen cells.

soluble chicken ovalbumin and 18-mer HDR or CpG-ODN in alum were used (data not shown).

Collectively, these studies were designed to explore the structure–immune function relationships of polynucleotides, on the basis of the presence (i.e., RNA) or absence (i.e., DNA) of the 2′OH on some or all of the respective bases, through the synthesis of defined HDR molecules. From these analyses we made a number of novel observations. (1) The substitution of even one of the six bases comprising the DNA "motif" with an analogous RNA base resulted in loss of TLR9-mediated function, thus underscoring the specific recognition requirements involved in TLR9 signaling. (2) We confirm that the region flanking the motif also plays a critical role in TLR9-mediated signaling by showing that either shortening the flanking sequence or changing the base composition leads to loss of activity. We then showed that, unlike the motif, this critical flanking sequence was functional either as RNA or DNA, but that the base itself, in this instance adenosine or deoxyadenosine was critical. (3) Regardless of whether the critical flanking sequence was RNA or DNA there was no significant change in the quantitative or qualitative activity, or TLR-specificity of the resulting sequences, thus underscoring the relatively permissive functional role of the flanking sequence, and the more specific role of the motif in mediating TLR9

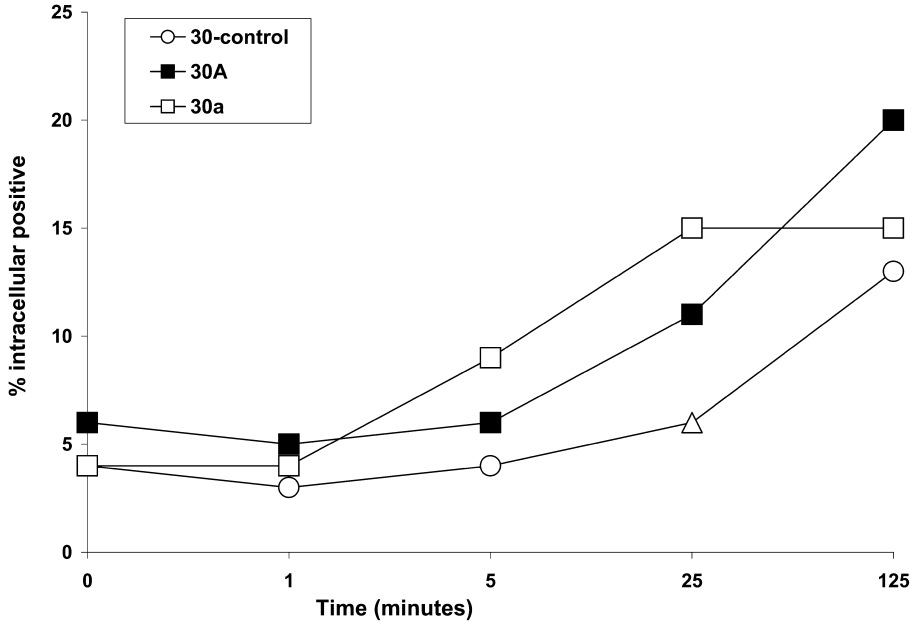

Fig. 7. Binding and internalization of HDR to spleen cells. BALB/c spleen cells were incubated with 1 μM of FITC-labeled 30-mer HDR, CpG-ODN or control HDR for 30 min at 4 °C or varying periods of time at 37 °C. The percent of cells that internalized the labeled HDRs was measured by FACS after quenching the surface bound labeled HDR with trypan blue. Similar results were obtained in three different experiments. Cells incubated on ice for 125 min with labeled ODN and cells cultured on ice for 30 min with labeled ODN, followed by washing with cold PBS, served as negative controls.

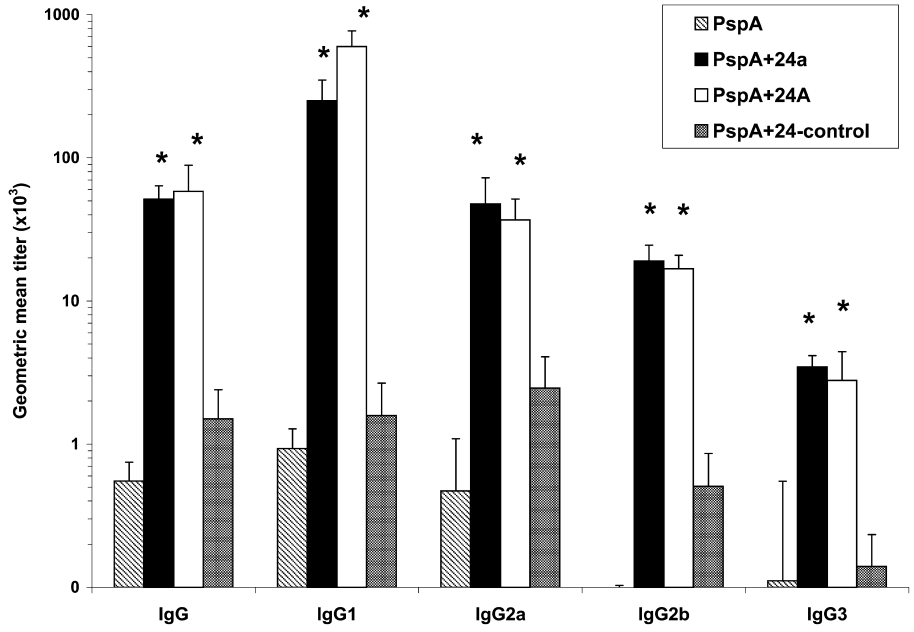

Fig. 8. HDR acts as adjuvant with enhancement of Th1-type antibody response. Group of seven BALB/c mice were immunized i.p. on days 0 and 28 with 0.5 μg of PspA adsorbed on 13 μg of alugel or in combination with 25 μg of HDR, control HDR or CpG-ODN. Immunized mouse sera obtained on day-35 and anti-PspA antibody was assayed by end point ELISA. OD value of one serum was used to construct standard curve and anti-PspA antibody titers were calculated from this standard curve. The geometric mean titer is shown for each group of seven mice (error bar indicates SEM). *Statistically significant results ($P < 0.05$) relative to geometric mean titer of only PspA immunized mice serum. These data are representative of three independent experiments with similar results.

signaling. Although HDR are unlikely to exist in nature, their use as an analytical tool has revealed some novel insights into the structural requirements important in TLR9-mediated signaling and a potential role for RNA in immunomodulation.

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